Comparison of Phosphoenolpyruvate-Carboxykinase from Autotrophically and Heterotrophically Grown Euglena and Its Role during Dark Anaerobiosis

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ABSTRACT

Euglena gracilis (1224-5/9) contains phosphoenolpyruvate carboxykinase when grown autotrophic with CO₂ in the light. Its yield is higher when an additional carbon source like glucose has been added. The enzyme is lacking in cells provided with CO₂ alone and kept in the dark, whereas highest yields result when both glucose and CO₂ are provided together in the dark. The enzyme was purified by ammonium sulfate precipitation, gel filtration on Sephacryl S-300 and affinity chromatography on GMP-Sepharose. The latter step was most effective to protect the enzyme from inactivation. Its homogeneity was tested electrophoretically and immunologically. Enzymes from autotrophic and heterotrophically grown cells have identical pH optima and similar isoelectric points. The molecular weight was different: 761,000 for the enzyme from autotrophic and 550,000 for that from heterotrophic cells as determined by gel filtration. The subunit molecular weight of both enzymes is nearly the same. The kinetic data of the enzymes are slightly different. Glycolytic and tricarboxylic acid cycle intermediates are of limited influence on enzyme activity and inhibitory in physiological high concentrations.

From Ouchterlony double immunodiffusion and enzyme-linked immunosorbent assay, it is evident that the enzyme is localized in the cytosol. With the latter quantification test the phosphoenolpyruvate carboxykinase protein content was found 10 times higher in heterotrophically grown cells than when cultivated under autotrophic conditions.

Euglena cells accumulate waxmonosesters in a high yield at the expense of their carbohydrate storage when they are kept autotrophic (10, 24, 26). For autotrophic waxester formation see Rosenberg (22). The occurrence of odd-numbered fatty acids and long chain alcohols in the lipid fraction pointed to the methylmalonyl-CoA pathway being involved and to propionyl-CoA as an intermediate (24). Incorporation studies with 14CO₂, succinate, and propionate support this view as well as the sensitivity of this kind of fermentation against rotenone (25). Further, from anaerobically supplied [2-14C]pyruvate no succinate was labeled (25). This implies that CO₂ fixation is not started from pyruvate. As Peak and Peak (19) failed to detect pyruvate carboxylase it seems rather sure that CO₂ fixation is started from PEP. (For a general discussion on CO₂ fixation, see Levedahl [14].) This paper deals with enzymes involved in CO₂ incorporation into oxaloacetate with PEP as the second substrate. PEP-carboxytransphosphorylase (4.1.1.38) seems to be absent in Euglena as demonstrated by Ohman and Plňák (18) and Peak and Peak (19) for strain Z and by Fischer (8) for strain T. PEPC (4.1.1.31) and PEPC (4.1.1.32), two other enzymes involved in PEP carboxylation, have been found in strain Z by Laval-Martin et al. (13) and by Peak and Peak (19). The latter authors and Miyatake et al. (17) failed to detect PEPC activity in autotrophically grown cells. We found both enzymes in our strain T, PEPC even in cells cultivated with CO₂ in light. The activities of these two enzymes depend strongly on growth conditions. In general PEPC activity involved in gluconeogenesis has been found to be higher when the cells were grown autotrophically or heterotrophically with lactate. PEPC is thought to function in anaplerotic CO₂ fixation in the dark in connection with amino acid biosynthesis (19), although a strict proof is still lacking.

A comparison of both enzyme activities in cells from autotrophic or heterotrophic growth in the dark showed that PEP-carboxylase could not be responsible for the high CO₂ incorporation rate described in our earlier papers. We therefore decided to follow up further PEP-carboxykinase only. Using autotrophically grown cells we followed the induction rate of PEPC in the light and in the dark in the presence of either CO₂ alone or glucose and CO₂. Additionally we purified the enzyme from autotrophic and heterotrophic cells and compared the properties of both proteins.

MATERIALS AND METHODS

Growth. Euglena gracilis T, strain 1224-5/9 (SAG, Göttingen, FRG) was cultivated either autotrophically (with 4% CO₂ in air) or heterotrophically with glucose (1%) as carbon source. The medium used was similar to that of Cramer and Myers (5). Cells were harvested at the end of the logarithmic phase of growth and checked routinely for bacterial contamination. Finally the cells were kept frozen at −20°C until use for enzyme purification.

Purification. All purification steps were carried out at 4°C. The cells were thawed and resuspended in extraction buffer (0.1 M Tris [pH 7.5], 5 mm MgCl₂, 5 mm MnCl₂, 5 mm DTT, 25 mm (NH₄)₂SO₄ and 0.5% [w/v] PVP) (4) and immediately disrupted by sonication using a Branson sonifier (step 5, cycle 20%, 12 min). After centrifugation first at 3,000g, 5 min and secondly at 40,000g, 20 min yielding the crude extract a protonate sulfate precipitation (final concentration 0.2% w/v) was followed. The clear supernatant of this step was subjected to (NH₄)₂SO₄ precipitation which was carried out in two steps: after the first precipitation at 25% the enzyme was still in the supernatant fluid, but with the subsequent step of 45% the enzyme was pelleted. After suspending the precipitated enzyme in extraction buffer (the
same buffer as above mentioned without PVP) it was immediately put on a calibrated Sephacryl S-300 (Pharmacia) (φ 2.5 x 86 cm) column which was previously equilibrated with the same buffer. The column was run with a flow rate of 28 ml/h and fractions of 2 ml were collected. Protein and enzyme activity were checked. Fractions containing more than 50% of the maximal PEPCK activity were pooled and applied to the next column which was an affinity chromatographic step using GMP-Sephrose. This material was prepared similar to the method described by Lamed et al. (12) and March et al. (16). The column (φ 1.6 cm x 32 cm) was equilibrated with 0.1 M Tris (pH 7.5) and 5 mM DTT was run with a flow rate of 21 ml/h. The pooled fractions from Sephacryl S-300 were applied to the column, washed with an appropriate amount of buffer, and eluted with a linear gradient of 0 to 3.75 mM MnCl₂ and ITP.

The GMP-Sephrose pool was directly used for kinetic analysis. For studying the homogeneity by gel electrophoresis the enzyme was previously concentrated by ultrafiltration.

Gel electrophoresis. Gel and buffer systems described by Davis et al. (6) were used for gel electrophoresis of native protein. SDS gel electrophoresis was done according to Studier (27) using acrylamide concentrations of 6 to 8% (w/v) acrylamide.

Protein. An aliquot of the samples was precipitated with TCA (0.5% w/v finally) and subsequently the protein was determined by the method of Lowry et al. (15) using BSA as a standard.

Preparation and partial purification of antiserum. Lyophilized enzyme protein (0.92 mg) was resuspended in complete Freund's adjuvant and finally injected intracutaneously into a rabbit. Injection was repeated after one week and subsequently at intervals of two to three weeks. A high titer of antibody was attained not earlier than six weeks after the first injection. The animal was bled to obtain approximately 30 ml of blood. The blood was left to clot for 2 h. Finally the antiserum was collected by centrifugation (40 min, 7700g), then (NH₄)₂SO₄ was added to a final concentration of 30%. The precipitate was centrifuged and it was redissolved in half the original volume of 0.15 M PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₃HPO₄, and 1.5 mM KH₂PO₄ (pH 7.2). The antiserum fraction was desalted on a PD-10 column (Pharmacia, Freiburg, FRG) previously equilibrated with 0.15 M PBS. Further purification of the serum was attained using a protein A-Sepharose column as described in the instruction manual "Affinity Chromatography" of Pharmacia (1).

Assay of enzyme activity. Following in principle the methods described by Briand et al. (3) both enzymes could be tested subsequently in the same vial. The reactions

\[
P + HCO₃⁻ \rightarrow \text{PEPC} \rightarrow \text{oxaloacetate}
\]

and

\[
P + HCO₃⁻ + GDP \rightarrow \text{PEPK} \rightarrow \text{oxaloacetate} + \text{GTP}
\]

were coupled to the reaction

\[
\text{Oxaloacetate} + \text{NADH} + H⁺ \rightarrow \text{malate} + \text{NAD}⁺ + \text{MDH}
\]

and could be followed spectrophotometrically at 360 nm.

In the direction of decarboxylation the labile substrate oxaloacetate was produced and was kept sufficiently constant by this auxiliary reaction. Routinely in decarboxylation 0.1 M Tris (pH 8.0) was used as described by Briand et al. (3) and in carboxylation 0.1 M imidazole pH 7.5. DTT (10 mM) was added in both directions. L-Malate was 20 mM and NAD-malate dehydrogenase was present with 10 units. For testing PEPC GDP (1.4 mM) or, for testing in the direction of decarboxylation, GTP (0.75 mM) was added. The reaction was started with the respective PEPC or PEPC preparations.

Propionyl-CoA carboxylase activity was tested as described by Kaziro (11).

**Immunoelectrophoresis.** Immunolectrophoresis (3.5 h; 100 V) was carried out in 1% agarose in 50 mM barbital buffer (pH 8.0). Better results were found if we used Tris-borate buffer (pH 8.5) (0.5% Tris, 89 mM borate, and 1 mM EDTA) instead of barbital buffer. Furthermore the presence of 0.1% Triton X-100 was necessary to facilitate diffusion of the enzyme.

ELISA. The test was carried out as described by Ponsgen and Betz (21) adapting it for the PEPC of Euglena. The antigen as standard was used in the range of 15 to 60 μg per 200 μl.

**RESULTS**

PEPC and PEPC Activities in Cells Grown Autotrophically and Heterotrophically. In cells grown auto- as well as heterotrophically, PEPC and PEPC could both be detected. The activities showed an inverse pattern (Table I). High PEPC activity could be observed during autotrophic growth, combined with low but clearly detectable PEPC activity. However, enlarged GDP-dependent PEPC-carboxylation was found during heterotrophy, where PEPC was low.

As PEPC is obviously the enzyme responsible for the incorporation of CO₂ in fermenting cells its induction rate was followed further in autotrophic and heterotrophic growing cells.

**Induction of PEPC.** When cells from autotrophic growth were transferred into the dark and supplied with glucose the activity of PEPC rose continuously with a lag period of nearly 12 h (Fig. 1a). During the exponential phase of growth PEPC was induced with a rate of 1.83 micromol h⁻¹. In the light with the same carbon source the rate of induction was similar but lower (81%, see Fig. 1b).

To determine whether proteins with PEPC activity are identical in autotrophic and heterotrophic Euglena cells, purification was made to study their properties.

**Purification.** Enzymes from both sources were purified near homogeneity with high yields (Table II and Fig. 2). The extent of purification was different: the specific activity of the protein from autotrophic cells was considerably lower than that from heterotrophic cells. With preparations from heterotrophic cells the enzyme was purified routinely to 70- to 100-fold with a yield of 10%.

From Figure 2, A and B, it is evident that the enzymes migrated in SDS-gel electrophoresis as a single protein band with almost the same subunit mol wt of 240,000. As marker proteins phosphorylase b (97,400), myosin (205,000), and β-galactosidase (116,000) were used.

**Immunological studies.** Antibodies were raised in rabbits against purified enzyme from heterotrophic cells. Using immunolectrophoresis we could show that the antibodies were pure and specific. Precipitin lines were found with both purified

| Table 1. PEPC-Carboxylating Enzymes in E. gracilis T |
|-----------------|-----------------|-----------------|
| Enzyme          | Heterotrophic   | Autotrophic     |
| Specific Activity | units/mg protein | units/mg protein |
| Crude extract   | 0.06            | 0.263           |
| PEPC            | 0.613           | 0.042           |
| Carboxylation   | 0.184           | 0.047           |
| Decarboxylation | 32.6            | 23.8            |
| Purified enzyme | 16.0            | 9.6             |
| PEPC            | 162.3           |

*Not determined.*
enzymes (Fig. 3). From these results it is evident that the two enzymes are not distinct immunologically. No precipitation was observed if control serum was used instead of antibodies.

In the immunoprecipitation test purified PEPCK was incubated with antibodies in various dilutions. The antigen-antibody complex was precipitated with protein A from Staphylococcus aureus. Figure 4 shows decreasing inhibition of enzyme activity with diluted antiserum. This result indicated an immunoreaction with the active protein.

In the ELISA test, crude extracts (supernatant of 40,000g) from autotrophically and heterotrophically grown Euglena cells were compared. The latter contained 48.8 ng PEPCK µg protein⁻¹, whereas only 2.5 ng/µg protein could be detected in autotrophic cells.

With protein fractions from mitochondria (containing 140–50 µg protein) from cells precultivated with malate-glutamate and finally grown in Koren-Hutner medium in the light no precipitin line could be detected in Ouchterlony double immunodiffusion.

Properties of PEPCK. Properties of both purified enzymes are summarized in Table III. Obviously they do not correspond in molecular size when determined on a Sepharose 4B gel filtration chromatography. On the other hand subunit mol wt as calculated by SDS-PAGE are nearly identical. The results of the size determination suggest that the heterotrophic enzyme is a dimer while the autotrophic one obviously consists of three equal subunits. The isoelectric points of the two enzymes are nearly identical. The same is true concerning the pH optimum of both: in the decarboxylation and in the presence of Mn²⁺ their values are exactly identical with a high value in the alkaline region; in the opposite direction(carboxylation) in the presence of either Mn²⁺ or Mg²⁺ the pH optima were also nearby identical (pH 7.5/7.6).

Most kinetic data of both purified enzymes are almost identical. However the Kₘ values for GDP and GTP are about 2-fold higher in the protein from heterotrophic cells. For HCO₃⁻ the Kₘ values of both enzymes differ only by a factor of 1.4. Nevertheless the Kₘ values for HCO₃⁻ are rather high. On the other hand both enzymes are characterized by low Kₘ values for oxaloacetate which indicates a strong binding of this substrate while the Kₘ for PEP is rather high in both proteins. In Table IV results of a series of experiments on the influence of metabolic intermediates, on the catalytic activity of PEPCK are summarized. It is surprising that the inhibitory effect of these organic acids is very low. Their role in regulating the enzyme activity cannot be important so much the more as high concentrations of presumed inhibitors have been used in this test. The limited but distinct effect of α-ketoglutarate both in decarboxylation and in carboxylation is probably due to the structural similarity between α-ketoglutarate and oxaloacetate. All other metabolites tested, including succinate, were of minor influence on PEPCK activity especially in the concentration range of physiological importance. Even in high concentrations (up to 20 mM), the inhibitory effect of these compounds was small.

With the exception of α-ketoglutarate the inhibitors summarized in Table IV were almost inefficient in the carboxylation reaction, too. The influence of α-ketoglutarate in different concentrations was further studied by varying either the concentra-

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**Table II. Comparison of Progress in PEPCK Purification between Cells either Grown Heterotrophic (A) or Autotrophic (B)**

<table>
<thead>
<tr>
<th></th>
<th>A. n = 6 (representative example shown)</th>
<th>B. n = 4 (representative example shown)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Activity</td>
<td>Protein</td>
</tr>
<tr>
<td></td>
<td>units</td>
<td>mg/ml</td>
</tr>
<tr>
<td>Crude extract</td>
<td>196.6</td>
<td>14.7</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>180.1</td>
<td>14.4</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>89.6</td>
<td>31.7</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>78.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>21.8</td>
<td>0.181</td>
</tr>
<tr>
<td>Crude extract</td>
<td>36.67</td>
<td>17.7</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>39.4</td>
<td>15.1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>15.9</td>
<td>41.2</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>16.5</td>
<td>0.77</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>2.6</td>
<td>0.13</td>
</tr>
</tbody>
</table>
FIG. 2. SDS-gel electrophoresis of purified PEPCK derived from heterotrophically (A) and autotrophically (B) grown cells. Protein (in the concentration range of 0.05–0.12 mg ml⁻¹) from pooled peak fractions after affinity chromatography on GMP-Sepharose was applied. Electrophoresis was carried out with 6% (A) or 6 to 8% acrylamide (B). (A) Lane 1 and 2: protein denatured for 2 and 5 min in a 100°C bath. Lane 3: myosin as standard (10 μg). (B) Lane 1 and 2: protein denatured as described above.

FIG. 3. Immunoelectrophoresis of purified PEPCK derived from cells grown heterotrophically (a and b, with 0.122 mg protein/ml) or autotrophically (c, d, and e, with 1.3 mg protein/ml). Enzyme extract (10 μl) was added to the wells and the channels 1, 2, 4, 5, and 6 were filled with antiserum (5.0 mg protein ml⁻¹) diluted 1:4 with PBS 0.15 M, whereas in channel 3 control serum was added.

The authors' conclusion that PEPCK is bound to heterotrophic growth, whereas PEPC is related to autotrophic conditions, is supported by our results. An inverse pattern of PEPC and PEPCK activity was further described for different growth phases of heterotrophic Euglena cells (on lactate) (3): PEPCK activity was high in the logarithmic phase and decreased when the cells became stationary. PEPC activity evolved just opposite.

Purification of PEPCK was accelerated by using affinity chromatography as the last step, which procedure additionally protected the enzyme from inactivation. Concerning yield and purification, our results correspond well with those described by Wicheavenonagoon and Arinze (29) for PEPCK from guinea pig mitochondria. They are rather different however from those described for yeast (28), and Ascaris (20).

After highest purification the specific activity of PEPCK from autotrophic Euglena is about 5 times lower than that from heterotrophic cells. This result fits well to that of immunooquan-
PHOSPHOENOLPYRUVATE CARBOXYKINASE FROM *E. GRACILIS* T.

Table IV. Influence of Metabolites on Activity of Purified PEPCK from Heterotrophically Grown Euglena Cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mM):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>36</td>
</tr>
<tr>
<td>Succinate</td>
<td>15</td>
</tr>
<tr>
<td>Fumarate</td>
<td>13</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>11</td>
</tr>
<tr>
<td>Malonate</td>
<td>13</td>
</tr>
</tbody>
</table>

The inhibition is expressed in percent of maximum activity using the decarboxylation reaction of the enzyme with a final Mn²⁺ concentration of 0.75 mM. A constant oxaloacetate concentration of 66 μM was produced by adding 20 mM L-malate.

FIG. 5. Double reciprocal plot of inhibition of PEPCK activity by α-ketoglutarate at variable PEP concentrations (a) and variable oxaloacetate concentrations (b), respectively. In (a), the concentration of MgCl₂ was maintained at 5 mM and MnCl₂ at 1 mM. In (b), a MnCl₂ concentration of 0.75 mM was used.

Fig. 5. Double reciprocal plot of inhibition of PEPCK activity by α-ketoglutarate at variable PEP concentrations (a) and variable oxaloacetate concentrations (b), respectively. In (a), the concentration of MgCl₂ was maintained at 5 mM and MnCl₂ at 1 mM. In (b), a MnCl₂ concentration of 0.75 mM was used.

Inhibition is expressed in percent of maximum activity using the decarboxylation reaction of the enzyme with a final Mn²⁺ concentration of 0.75 mM. A constant oxaloacetate concentration of 66 μM was produced by adding 20 mM L-malate.

Free sulfhydryl groups are essential for optimal catalytic activity of Euglena PEPCK. DTT was used in our assay system. Sulfhydryl binding compounds are strong inhibitors. Most effective were p-hydroxymercuriphenyl sulfonic acid and ethylmal- eimide. The inhibitor concentration for more than 50% inhibition is variable from 10⁻³ to 2 × 10⁻⁶ M dependent on the age of the purified enzyme. DTT or glutathione failed to prevent this inhibitory effect.

The pH optimum for both directions of PEPCK activity was found in the alkaline region. This is in contrast to Miyatake et al. (17) who described a more acid optimum for carboxylation for his strain Z. The kinetic data of both enzymes are similar to those of proteins from vertebrate and invertebrate systems. Moreover they correspond with the kinetics determined from another strain of Euglena (Table V). Most striking among all these data is the difference in $K_m$ for HCO₃⁻. This value is extremely high in chicken liver enzyme (9) and surprisingly low in Euglena strain Z. The $K_m$ values for the Ascaris enzyme and for our strain are intermediate.

From these $K_m$ values a functional difference is evident between PEPCK from chicken, Ascaris, and Euglena (strain T). Its low affinity to HCO₃⁻ points to oxaloacetate decarboxylation in the enzyme from chicken liver. In Ascaris, carboxylation seems to be the preferred direction (20): the $K_m$ for HCO₃⁻ is 3-fold lower than in the chicken enzyme and its affinity to PEP is higher than to oxaloacetate. Carboxylation is additionally favored by anaerobic conditions in the parasite's environment.

PEPCK from our Euglena strain seems well suited to catalyze anaerobic CO₂ fixation. We expect that it is the key enzyme for the methylmalonyl-CoA pathway yielding odd-numbered chains of fatty acids and alcohols by incorporation of propionyl-CoA into waxynoesters in fermenting Euglena (24–26).

The enzyme from autotrophic cells has a $K_m$ for HCO₃⁻ of 4 to 5 mM at pH 7.5. This corresponds to a CO₂ concentration of 0.3 to 1.2 mM in vivo (2). Rising CO₂ concentrations in anaerobic cells favour carboxylation (24). As calculated from the data of Schneider and Betz (26) odd-numbered fatty acid and alcohol chains are synthesized with about 0.016 mM min⁻¹. In earlier studies on CO₂ incorporation (26) into odd-numbered chains a rate of 0.22 nmol min⁻¹ mg protein⁻¹ was found for propionyl-CoA formation. This corresponds well with an activity of propionyl-CoA carboxylase of 0.33 milliunit × mg protein as found in crude extract (data not shown). An activity of PEPCK in the direction of carboxylation of 42 milliunits mg protein (Table I) seems high enough to catalyze the first step in anaerobic CO₂ fixation tests which showed that 20-fold more PEPCK protein was synthesized in heterotrophic growth than in autotrophy. Both observations support the idea that this enzyme is related mainly to heterotrophy.

The mol wt of Euglena PEPCK was found distinctly higher than in the enzymes from yeast (28), Ascaris (20), or that from guinea pig liver mitochondria (29). The heterotrophic protein of Euglena can be split into two subunits, the autotrophic one in three with almost identical mol wt of the subunits (240,000–247,000) in both forms (Fig. 2, A and B). Their high values are most striking, but the similarity to the multi-enzyme complex described by Ernst-Fonberg and Wolpert (7) could be excluded, as no PEP activity was present after affinity chromatography.

For optimal catalytic activity the presence of divalent cations is absolutely required. Most effective are Mn²⁺ and Mg²⁺. When tested in the direction of carboxylation Mg²⁺ was 5 mM and Mn²⁺ 1 mM in the presence of 1.4 mM GDP in our experiments. Activation of PEPCK by a combination of Mn²⁺ and Mg²⁺ agrees with Satoh's (23) results with embryonal chicken liver. With our enzyme decarboxylation was tested with 0.75 mM Mn²⁺ only. In summary a common feature of all the PEPCKs purified so far from different sources is their stimulation by divalent cations.

Table V. Comparison of Kinetic Parameters of Purified PEPCK from Different Sources

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Ascaris</th>
<th>Chicken</th>
<th>E. gracilis Z</th>
<th>E. gracilis T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$K_m$</td>
<td>$K_m$</td>
<td>$K_m$</td>
</tr>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>7.1</td>
<td>20.0</td>
<td>0.066</td>
<td>4.0</td>
</tr>
<tr>
<td>PEP</td>
<td>0.12</td>
<td>0.155</td>
<td>0.13</td>
<td>0.8</td>
</tr>
<tr>
<td>GDP</td>
<td>0.022</td>
<td>0.051</td>
<td>0.059</td>
<td>0.056</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>0.84</td>
<td>0.014</td>
<td>0.016</td>
<td>0.022</td>
</tr>
<tr>
<td>GTP</td>
<td>0.065</td>
<td>0.065</td>
<td>0.141</td>
<td>0.065</td>
</tr>
</tbody>
</table>
incorporation.

Our kinetic data on PEPCK fit well to the observations on the induction of the enzyme, as described in this paper, as well as to older data on 14C incorporation into waxesters in fermenting Euglena cells (23, 26) and to the results of Laval-Martin (13).

The problem of how PEPCK is controlled in living cells is still open. All metabolic intermediates so far tested are of minor efficiency. No significant influence can be expected under physiological conditions (Table IV). Only a-ketoglutarate was found slightly inhibitory in both directions, obviously in competition to oxaloacetate. For all PEPCKs purified thus far, no indications are known for control by glycolytic or tricarboxylic acid cycle intermediates. The differences between heterotrophic and autotrophic forms of PEPCK point to control at enzyme expression.

The localization of PEPCK in E. gracilis is still under discussion. Using antibodies against the heterotrophic form of the enzyme we found it exclusively in the cytosol of heterotrophic cells which had been raised in Koren-Hutner medium. Miyatake et al. (17) described the same for their strain Z with cells grown in the same medium under illumination. In contrast Briand et al. (3) postulated it to be localized in the mitochondrial matrix.

Acknowledgments—We thank Mrs. H. Geithmann for photographic work and Mrs. C. Borkowski for preparation of mitochondria.

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